Cell, Vol. 103, 997–1000, December 22, 2000, Copyright ©2000 by Cell Press

# Waiting for Anaphase: Mad2 and the Spindle Assembly Checkpoint

Minireview

**Jagesh V. Shah* and Don W. Cleveland*†‡**
*Department of Cellular and Molecular Medicine
†The Ludwig Institute for Cancer Research
University of California at San Diego
La Jolla, California 92093

The major function of the mitotic spindle is to ensure that when cells divide each daughter faithfully receives one copy of each chromosome. For this to happen, spindle microtubules first attach to kinetochores, the proteinaceous complexes assembled at the surface of the centromeres of each chromosome. Microtubule motors at the kinetochores (along with others, named chromokinesins, bound to the chromosome arms) power chromosome congression to an aligned configuration, called metaphase. Subsequently, the main mitotic business ensues: the degradation of the glue holding duplicated sister chromosome pairs together, and the kinetochore-directed movement of one copy of each chromosome to each spindle pole, a process referred to as anaphase.

Fidelity is key: chromosomes lost along the way generate aneuploidy, an event generally lethal during development and a feature of the aberrant growth regulation associated with tumor progression. Faithful transmission of chromosomes is mediated by a spindle assembly checkpoint (sometimes called the mitotic checkpoint) that acts to prevent anaphase until all kinetochores have successfully captured one or more spindle microtubules, thus ensuring that no chromosome is left behind, either at mitosis or meiosis. The spindle checkpoint represents a highly conserved mechanism at work in many cell types from single-celled organisms through metazoans.

The basic plan of the signaling cascade is now well established. Central to the spindle checkpoint is the kinetochore. Prior to spindle attachment, kinetochores generate a diffusible "wait anaphase" signal, which inhibits the anaphase promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase associated with proteosome-mediated proteolysis. When associated with appropriate specificity factors (e.g., cdc20 in yeast, fizzy in *Drosophila*, or p55cdc in mammals), APC/C ubiquitinates proteins whose degradation is required for the onset of anaphase, such as the securins (aka Pds1p in budding yeast), which are essential for maintaining the glue holding the sister chromatids together, and chromokinesins (Figure 1). As a kinetochore binds microtubules (a single microtubule in yeast, but up to 30 in mammals), its wait signal generator is silenced and the inhibition of anaphase is released.

The initial mechanistic insight into the components that contribute to checkpoint signaling arose from two similar genetic screens in yeast for mutants that do not arrest in mitosis following drug-induced inhibition of spindle microtubule assembly. These identified two trios of nonessential genes in budding yeast, Bub1-3, for budding uninhibited by benzimidazole and Mad1-3, for mitotic arrest deficient. To these has been added Mps1. This kinase is essential for duplication of the microtubule nucleation sites (called spindle pole bodies in yeast), but mutations in it also bypass the spindle checkpoint (see Amon, 1999, for references).

However, several central questions remain. What is the composition of the "wait anaphase" signal and how does it prevent anaphase onset? How does chromosome attachment to the spindle turn off the signal generator and what cytoplasmic events deactivate the already active wait signal? As we detail below, the answers to many of these key questions revolve around Mad2, an abundant protein complexed with the ubiquitination/proteolytic degradation machinery and found at the kinetochores of chromosomes that are not yet attached to the spindle—properties that make Mad2 perfectly poised to deliver signals for the spindle checkpoint.

### *Mad2, a Part of the Diffusible "Wait Anaphase" Signal*

Considering the minimal elements required to construct such a checkpoint, McIntosh (1991) initially proposed kinetochores themselves as the signal generators. This was firmly established by a pair of elegant experiments by Rieder and colleagues showing that a single kinetochore, when prevented from attaching to the spindle, inhibited anaphase. Furthermore, ablation of that lone unattached kinetochore using a laser resulted in the rapid onset of anaphase (Rieder et al., 1995). This provided clear evidence that the unattached kinetochore produced a diffusible "wait anaphase" signal and that this signal, and its silencing by spindle attachment, was the key event in satisfying the mitotic checkpoint.

Further insight into the checkpoint mechanism was provided through the demonstration that the homologs of Mad2 in *X. laevis* and humans are enriched at unattached kinetochores, as are Bub3, Mad1, and the mitotic kinases Bub1, MAP kinase, and BubR1 (the mammalian Mad3) (see Amon, 1999, for references). Most recently, immunoprecipitation and immunoblotting has shown soluble Mad2 to be bound either to cdc20, to the APC/C, or to both (e.g., Hardwick et al., 2000). Current models (Figure 1) propose that Mad2 is activated (denoted here as Mad2*) by associating with unattached kinetochores. The subsequent release of Mad2* provides the diffusible signal (a reporter of unattached kinetochores) that directly interacts with the cell cycle proteolytic machinery to prevent the onset of anaphase.

In a report last October (Howell et al., 2000), Salmon and colleagues tested the core premise of this model, that is, does Mad2, in fact, dynamically associate with unattached kinetochores? Mitotic rat kangaroo ($PTK_1$) cells were microinjected with fluorescently marked Mad2 and the subsequent fluorescence at one unattached kinetochore was photobleached with a focused laser. Fluorescence was found to recover with a very rapid half recovery time ($t_{1/2}$) of ~24–28 s, presumably

‡To whom correspondence should be addressed (e-mail: dcleveland@ucsd.edu).

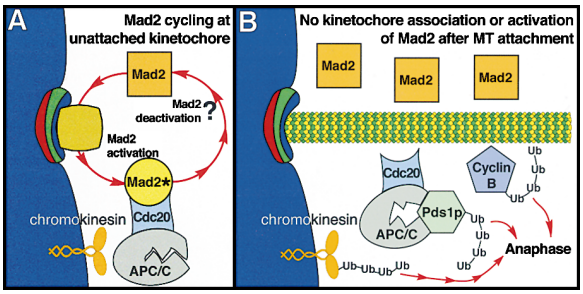


Figure 1. Activation of Mad2 at Kinetochores Regulated by Microtubule Attachment

The activation of Mad2 at unattached kinetochores is posited to occur through a binding/release model in which Mad2 is "activated", becoming Mad2*, which is then released into the cytoplasm.

(A) Mad2* prevents the onset of anaphase by inhibiting the cdc20-anaphase promoting complex/cyclosome complex.

(B) Upon microtubule attachment, Mad2 is no longer recruited to the kinetochore and Mad2* is no longer generated, releasing inhibition of cdc20-APC/C and initiating anaphase onset through ubiquitination and degradation of securin/Pds1.

reflecting the dynamic release of bleached Mad2 and its quick replacement from the soluble, fluorescent Mad2 pool. This recovery time was indistinguishable from that of a GFP-tagged human Mad2 introduced by transfection and both recoveries were shown to be ATP dependent. The demonstration of rapid turnover offers strong support for the catalytic turnover model of Mad2 activation (Figure 1) and provides a plausible basis for the ability of a single unattached kinetochore to block anaphase onset.

But what is Mad2*, and how does the kinetochore generate and release it? Attempts to identify posttranslational modifications of Mad2 that may represent Mad2* have all been unsuccessful. However, Fang and colleagues have proposed that inhibition of cdc20-APC/C may occur via an oligomerized form of Mad2 (Fang et al., 1998). This hypothesis arose from identifying a species of Mad2 produced by expression in bacteria. With characteristics of a tetramer, this form of Mad2 was more potent than monomeric Mad2 in blocking APC/C ubiquitination activity and in arresting *Xenopus* embryos and egg extracts in mitosis. The oligomer model proposes that the kinetochore acts as a site of assembly for Mad2 oligomers that then quickly release as Mad2*. As microtubules attach to kinetochores, Mad2 is no longer recruited to kinetochores and thus, less oligomeric Mad2 is generated.

Although this model is attractive, we should emphasize that no in vivo evidence yet supports it. First, no checkpoint-dependent Mad2 oligomer generation has yet been identified in vivo. Second, the composition of Mad2* may not be so simple as the oligomer model posits. Evidence in budding yeast has revealed that Mad2 binds a number of other spindle checkpoint proteins, including Mad3 and Bub3, which are all apparently complexed with cdc20 (Hardwick et al., 2000). This implicates a larger wait signal complex. Third, although it seems very likely, we do not know if the Mad2 that is turning over at kinetochores represents the conversion of Mad2 to Mad2*.

### *Mad2 and the Checkpoint Are Essential in Metazoans*

The original description of the mitotic checkpoint in yeast defined a nonessential pathway invoked only in response to damage. But in metazoans, the checkpoint has evolved into an essential feature of normal mitoses and meioses. Microinjection of antibodies to Mad2 into cultured animal cells first revealed premature anaphase onset and chromosome missegregation (Gorbsky et al., 1998), presumably by antibody sequestration of Mad2 (and/or Mad2*) away from the kinetochore or the cdc20-APC/C complex. In the whole animal, RNA interference of the *C. elegans* Mad2 homolog (Mdf-2) showed that loss of Mad2 resulted in an embryonic or larval arrest in ~20% of animals (Kitagawa and Rose, 1999). The remaining animals developed to the adult stage but displayed dramatic defects in germ line development, underscoring the role of Mad2 in the meiotic spindle checkpoint.

In the mammalian context, loss of Mad2 in mice is lethal early, with defects arising by embryonic day 6.5 (Dobles et al., 2000). In null embryos, abnormal chromosome segregation and nicked DNA are found, consistent with an apoptotic cell death presumably acting to remove aneuploid cells. Preliminary analysis of the heterozygotes indicates an intriguing increase in tumor incidence. This last finding is, no doubt, just the initial foray in documenting how compromised Mad2-dependent signaling may be reflected in tumor progression.

### *Silencing Checkpoint Signaling: Tension or Attachment of Microtubules?*

The deactivation of Mad2* is also not understood. By following fluorescently tagged Mad2 in live cells, Howell et al. (2000) showed that anaphase initiates ~10 min after Mad2 is no longer detectable at any kinetochore. Since this time is shorter than the ~20 min required for anaphase onset after ablation of, or microtubule attachment to, the last unattached kinetochore (Rieder et al., 1995), the 10 min probably represent the actual time required to deactivate the active cytoplasmic pool of Mad2*. What is clear is that the signal diffuses from unattached kinetochores (how else can unattached kinetochores send a signal?). Nevertheless, in fused $PTK_1$ cells with two spindles in the same cytoplasm, anaphase can initiate in one spindle even if the other has unattached chromosomes (Rieder et al., 1997). Presumably, this reflects competition between the short half-life of Mad2* and its finite diffusion rate from the last unattached kinetochore(s). Once anaphase starts in one spindle, however, the other spindle also proceeds to anaphase irrespective of any remaining unattached kinetochores.

Beyond Mad2* deactivation, a controversy exists as to whether silencing of the signal generator is due to microtubule attachment to kinetochores or the subsequent tension developed across bi-oriented kinetochore pairs. In a meiotic context, using microneedles to manipulate chromosomes in insect spermatocytes, Li and Nicklas (1995) demonstrated that failure of the Y chromosome to pair with the X in meiosis I yielded a mono-oriented Y that could stall advance to anaphase, but anaphase was triggered shortly after mechanical application of tension across the mono-oriented kinetochore. This then led to Nicklas's meiotic error correction model

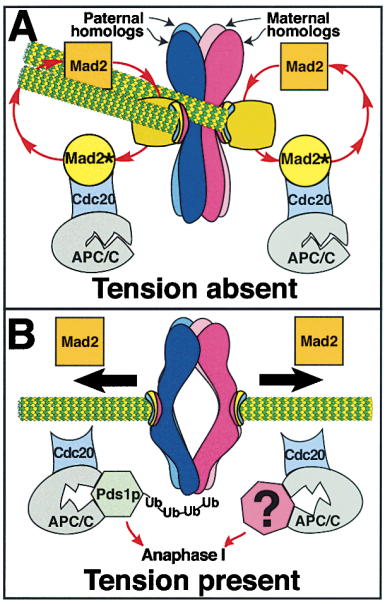


Figure 2. Mad2 Activation in Meiosis I May Be Regulated by Tension

Mad2-dependent checkpoint signaling in meiosis may be silenced by mechanical tension at kinetochores. Microtubule capture by the kinetochores on homologous chromosome pairs in meiosis I is not sufficient to satisfy the checkpoint, possibly because (A) microtubules from one pole can simultaneously bind to kinetochores on both pairs of chromosomes. (B) Correct bi-orientation allows development of tension that blocks Mad2 recruitment and activation, silencing the checkpoint.

in which tension silences the wait anaphase signal only when homologous pairs are attached to the different spindle poles, i.e., when tension is present (Figure 2). But this model cannot be universal, since other insect species manage meiosis normally with mono-oriented chromosomes. Moreover, in $PTK_1$ cells, loss of Mad2 staining at kinetochores, when the checkpoint is presumed to be off, depends on microtubule attachment, not tension (Waters et al., 1998).

The resolution to this controversy arose from a study in maize (Yu et al., 1999) revealing, diplomatically, that both sides of the controversy are right. Loss of the plant homolog of Mad2 at kinetochores coincides in mitosis with microtubule attachment, but in the same organism during meiotic divisions, loss of kinetochore Mad2 correlates with tension, not just microtubule binding. Binding and tension are probably best viewed as two sides of the same coin, with tension in meiosis now seen as the norm.

Indeed, in a clever set of experiments, chromosome segregation in budding yeast meiosis was visualized directly by tagging one chromosome using integrated LacO DNA arrays and stably expressed GFP-LacI. Disruption of the Mad2 gene resulted in defective chromosome segregation predominantly in meiosis I, indicated by many spores with an inappropriate number of fluorescent dots (Shonn et al., 2000). Eliminating tension between homologs, by preventing recombination (using a spo11Δ mutant), resulted in a dramatic delay in checkpoint silencing. This delay was eliminated by forcing individual homologs to biorient (using a spo11Δspo13Δ

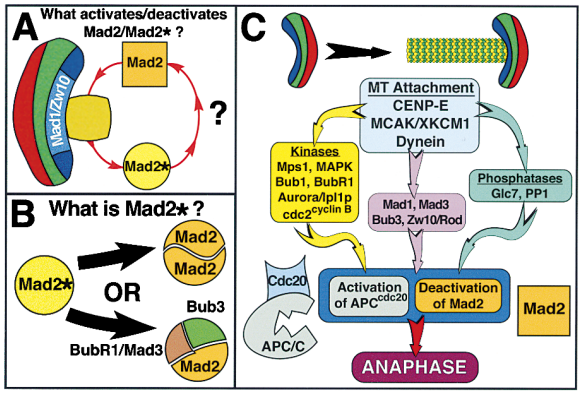


Figure 3. Unresolved Questions in the Spindle Checkpoint

Features of checkpoint signaling yet to be experimentally verified include (A) what is Mad2*, (B) what deactivates it, and (C) how do the components upstream of Mad2 generate Mad2* at unattached kinetochores and how is such signaling silenced upon microtubule attachment?

mutant), thereby restoring tension and demonstrating that checkpoint silencing requires the ability to generate tension across bi-oriented kinetochores.

***What "Makes" and "Breaks" the Wait Signal?***

Although all the pieces of the puzzle may seem to be in place, there are still several key processes that have been widely posited but remain experimentally unverified. What attracts Mad2 to the kinetochore and what causes its release? Is it Mad1, as has been hypothesized from its apparent loss (along with Mad2) from the kinetochore after microtubule attachment (Figure 3A)? A pair of reports reveal a fly in the ointment (Basto et al., 2000; Chan et al., 2000). Specifically, they demonstrate that the zeste-white 10 protein (Zw10) and its binding partner, rough deal (Rod), both originally discovered in *Drosophila* and without homologs in yeast, are required for activation of the spindle checkpoint in human cells and flies. These proteins have been previously shown to recruit dynein and dynactin to the kinetochore and, upon microtubule attachment, to "stream" along kinetochore microtubules to the spindle poles—an attractive mechanism for turning the checkpoint off. So it comes as a big surprise that disruption of Zw10 or Rod function genetically (Basto et al., 2000) or with antibodies (Chan et al., 2000) did not arrest cells in mitosis but instead prevented cells from activating the checkpoint in the presence of spindle damage. In human cells, antibody inhibition of Zw10 or Rod function leaves Mad2, the trusty wait signal, still present at the kinetochores of the unattached chromosomes even though the checkpoint is off (Chan et al., 2000)!

How could this be? One possible interpretation is that some proteins may be required to bring Mad2 to the kinetochore (e.g., Mad1), whereas others, Zw10 or Rod for example, may be responsible for releasing Mad2*. If this is the case, Zw10 and Rod must only be important for releasing Mad2* from unattached kinetochores because Mad2 is released from attached kinetochores despite the loss of Zw10 and Rod (Figure 3A). This hypothesis is now directly testable—does Mad2 fail to cycle at unattached kinetochores in Zw10 or Rod disrupted cells?

Determining the components of the inhibitory signal (Figure 3B) will firmly establish the molecular link between the unattached kinetochore and the inhibition of anaphase. The bigger question, of course, is how these elements are coordinated upstream of Mad2 and downstream of spindle attachment (Figure 3C). Attachment of microtubules to the kinetochore is likely to be mediated by dynein and kinesin motors (e.g., CENP-E, XKCM1/MCAK; for review see Sharp, et al., 2000), especially CENP-E, a kinetochore kinesin that can directly bridge between spindle microtubules and the checkpoint kinase BubR1 (Chan et al., 1999; Yao et al., 2000) and which can be essential for kinetochore signaling in vitro (Abrieu et al., 2000). Microtubule attachment leads to even more cascades that will undoubtedly involve mitotic kinases such as Mps1, MAP kinase, Bub1, and BubR1 and the little talked about phosphatases such as budding yeast Glc7/PP1. In addition, proteins such as Bub3, Mad3, Mad1, and Zw10/Rod all play an as yet undefined role in the generation, release, regulation, and/or composition of the wait signal. Stay tuned. Much more remains to be unraveled.

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